FEBS Letters 581 (2007) 4645–4650

Floating culture promotes the maintenance of hematopoietic stem cells

Teruyuki Kajiume[a,*], Louis Yuge[b], Yumi Kawahara[b], Reiko Yoshimoto[b], Akira Sasaki[b], Toshinori Ide[c], Makoto Asashima[d], Katsuko Kataoka[e], Masao Kobayashi[a]

[a] *Department of Pediatrics, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*
[b] *Department of Bio-environmental Adaptation Sciences, Graduate School of Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*
[c] *Department of Cellular and Molecular Biology, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*
[d] *Department of Life Sciences, Graduate School of Arts and Sciences and International Cooperative Research Project (ICORP), Japan Science and Technology Corporation (JST), The University of Tokyo, Tokyo, Japan*
[e] *Department of Histology and Cell Biology, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan*

Received 15 June 2007; revised 22 August 2007; accepted 24 August 2007

Available online 7 September 2007

Edited by Ned Mantei

Abstract In this study we examined the effect of the specific gravity of culture medium on the frequency of hematopoietic stem cell (HSC) maintenance. We used a newly developed high-specific-gravity media. Bone marrow cells were isolated and cultured, and HSC activity was evaluated. The number of hematopoietic progenitor/stem cells was markedly higher in the medium with high specific gravity. In high-specific-gravity media, cells did not precipitate, maintenance of HSCs was increased, and there was a concomitant accumulation of β-catenin. This novel technique for maintaining HSC populations provides an important new tool for studies in regenerative medicine.

Keywords: Hematopoietic stem cells; Specific gravity; Regenerative medicine

1. Introduction

The differentiation of hematopoietic stem cells (HSCs) has become an intense area of research in the field of hematology, and the hematopoietic microenvironment is known to be essential to HSC survival. The alternative ''niche'' hypothesis is also important and was first proposed many years ago after hematopoietic cells were cultured on bone marrow stromal cells [1,2]. We demonstrate here that HSCs can survive *in vitro* under specific conditions.

HSCs are usually cultured in medium with a specific gravity of approximately 1.004 (g/mL). Thus, cells precipitate to the bottom of the dish. Hematopoietic cells are drifting cells, which do not precipitate *in vivo*. We therefore hypothesized that an environment comprising floating hematopoietic cells would be closer to the true bone marrow microenvironment. To address this, we cultured hematopoietic cells in media of various specific gravities, to allow the cells to float to varying degrees. We found that specific gravity affected the maintenance of HSCs.

*Corresponding author. Fax: +81 82 257 5214.
E-mail address: kajiume@hiroshima-u.ac.jp (T. Kajiume).

2. Materials and methods

2.1. Mice

Five- to 8-week-old C57BL/6 (Ly5.1 and Ly5.2) mice were used in this study. Bone marrow transplantations were performed using green fluorescent protein-transgenic (GFP tg) mice [3] as the donor mice. All mice were bred and maintained in the animal facility at Hiroshima University.

2.2. Marrow cells cultured in each of the media with different specific gravities

Bone marrow was flushed from the medullary cavities of the bones with Ca^{2+}-/Mg^{2+}-free phosphate-buffered saline (PBS). The basic culture medium which we used is Dulbecco's modified Eagle's medium DMEM; (Amersham Biosciences Corp., Piscataway, NJ). The specific gravity of each of these media was adjusted using Percoll (Amersham Biosciences Corp.). In addition, STEMPRO-34 Nutrient Supplement (Catalog number 10641; Invitrogen, Carlsbad, CA) and cytokines are added in this medium. Human flt-3-ligand (PeproTech, London, UK) and human thrombopoietin (Kirin Brewery Co, Tokyo, Japan) were added to each medium at a final concentration of 20 ng/mL. Bone marrow cells (1.0×10^6 cells/well) isolated from mouse were cultured in the media with various specific gravities, in 24-well plates, with 1 mL of medium in each well. The culture plates were incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 7 days. Purified lineage marker-negative, Sca-1-positive, and cKit-positive (LSK) cells were used for the experiments shown in Fig. 3. Whole bone marrow cells were used in all other experiments.

2.3. Flow cytometric assay and colony-forming unit (CFU) culture

To sort and analyze the HSCs, the bone marrow-derived cells were labeled with an antibody cocktail consisting of biotinylated anti-Gr1, -Mac1, -B220, -CD4, -CD8, and -Ter119 antibodies as lineage markers, as well as streptavidin-PerCP. The cells were also stained with phycoerythrin (PE)-conjugated anti-Sca1 and allophycocyanin (APC)-conjugated anti-ckit antibodies. All antibodies were purchased from PharMingen (San Diego, CA). Dead cells stained with propidium iodide were excluded. The cells were analyzed using a FACS Caliber system, and sorted with a FACS Vantage system (BD Biosciences, Bedford, MA). Sorted cells with a purity of at least 95% were used for further experimentation.

The *in vitro* colony-forming cell (CFC) activity was assessed using a methylcellulose colony assay (Methocult GF M3434; StemCell Technologies, Vancouver, BC, Canada).

2.4. Bone marrow transplantation

Prepared cells (corresponding to 2×10^5 GFP marrow cells after culturing) from the donor mice were transplanted intravenously into lethally irradiated (9 Gy) C57BL/6 Ly5.2 recipients. For the competitor

0014-5793/$32.00
doi:10.1016/j.febslet.2007.08.057

cells, 2×10^5 marrow cells from the Ly5.1 mice were used. Peripheral blood samples were collected from recipient mice 16 weeks following transplantation.

2.5. Immunohistochemistry and Western blotting

After 7 days in culture, the marrow cells were fixed with 4% paraformaldehyde and stained with antibodies to β-catenin (Zymed Laboratories, Minneapolis, MN) and N-cadherin (IBL, Gunma, Japan). FITC-conjugated goat anti-mouse IgG and Alexa 594-conjugated goat anti-rabbit IgG were used as secondary antibodies for the immunostaining, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was used for Western blotting.

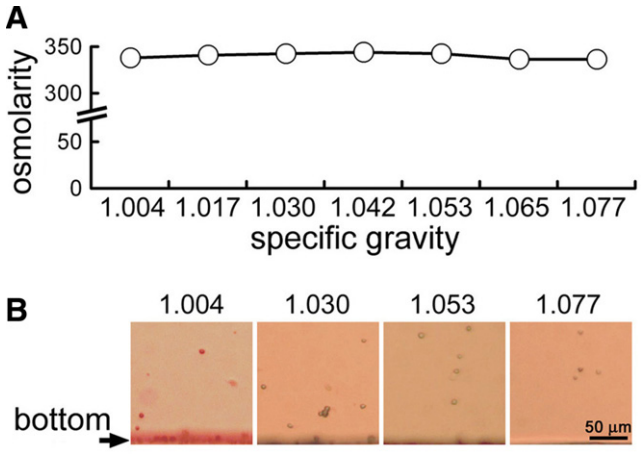

Fig. 1. Culture media characteristics. (A) All culture solutions had the same osmolarity. (B) Side view showing the specific gravity at which the cells precipitated. The marrow cells did not precipitate in the high-density medium (specific gravity 1.053). The cells floated completely in medium with a density of 1.077.

3. Results

3.1. Optimization of specific gravity of medium and cell culture schedule

Initially, we determined which cytokines were essential for the culture of bone marrow cells. Although the final number of cultured marrow cells was reduced with all combinations of cytokines (stem cell factor, thrombopoietin (TPO), and flt3-L), the combination of TPO and flt3-L caused the least decrease and was suitable for maintaining the precursor cells (data not shown). The combination of TPO and flt3-L was therefore used throughout this study.

The specific gravity of the solution comprising standard DMEM, supplements, and cytokines was 1.004. We prepared media with six specific gravities up to 1.065 and cultured mouse marrow cells for 7 days. The osmolarity of these culture solutions did not change (Fig. 1A). The marrow cells did not precipitate at or above the density of 1.053 (Fig. 1B); on the third and seventh days many cells remained floating in the medium with specific gravity 1.053, which was used as the highest density medium in subsequent experiments (Fig. 2A).

LSK cells possess most of the long-term multilineage reconstitution activity [4–6]. On culture day 7, an increased number of LSK cells was present in the high-density medium (Fig. 2B). The flow cytometric profiles are shown in Fig. 2C. A methylcellulose colony assay identified the functional hematopoietic cells. The culturing schedule is shown in Fig. 2D. The number of CFCs was also amplified in the high-density medium (Fig. 2E), as observed with the LSK cells.

We purified the LSK cells to examine whether HSCs were maintained, and cultured them by the same procedure. The first 100 LSK cells were cultured for 7 days. The LSK cells cul-

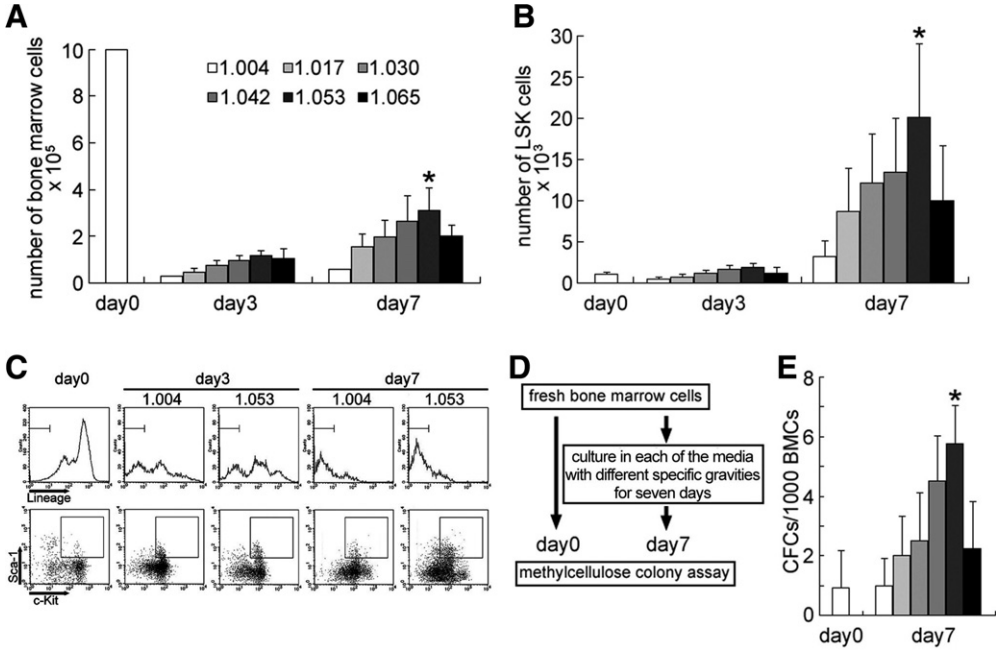

Fig. 2. Proliferation of bone marrow cells and progenitor cells in media of different specific gravities. (A) Total cell numbers on the third and seventh days of culturing. Although the number of cells decreased temporarily because differentiated cells exist in the bone marrow, many cells remained in the high-density medium (specific gravity of 1.053). (B) The number of LSK cells was amplified in this medium. (C) Flow cytometric profiles in each of the media having different specific gravities. After seven days of culture, the number of LSK cells had increased in the high-density medium. (D) Culturing schedule for the methylcellulose colony assay. (E) The number of CFCs was similarly amplified in the high-density medium. The difference in amplification was marked.

tured in the lowest-density medium (specific gravity 1.004) did not survive. However, the number of purified LSK cells increased approximately 10-fold in the high-density medium and almost all cells were LSK cells (Fig. 3).

3.2. Functional analysis of HSCs using in vivo bone marrow transplantation

We performed bone marrow transplantations to assess the functional status of the amplified HSC cells (LSK and CFC), using the GFP tg mice as donors and marrow cells from Ly5.1 mice as competitors. Equivalent numbers of GFP marrow cells were transplanted into lethally irradiated Ly5.2 mice (9 Gy). Subsequently, the GFP cells cultured for 7 days in the various media were transplanted, and the outcomes were compared 16 weeks after transplantation, by sampling peripheral blood cells from the recipient mice. GFP-positive cells were detected by flow cytometry. While cells cultured in the lowest-density medium were not well retained by the irradiated recipient mice, those grown in the high-density medium (specific gravity 1.053) were retained in high numbers (Fig. 4A, B), and therefore at day 7 post-transplant remained as functional HSCs. This transplantation experiment was performed three times, and the results are summarized in Table 1. The results indicated that the surviving cells in the high-density culture medium remained as functional HSCs at the end of 7 days.

3.3. Molecular mechanism underlying HSC maintenance

Bone marrow cells did not precipitate readily in media with a high specific gravity. β-Catenin is an important mediator of cell adhesion, cytoskeletal function, and HSC maintenance [7,8]. We therefore examined β-catenin and another adhesion protein, N-cadherin, in the marrow cells by immunostaining on the seventh day. N-Cadherin expression was increased, which was contrary to our expectation in media containing many floating cells. Additionally, β-catenin accumulation was increased, in comparison with cells cultured in the lowest-density

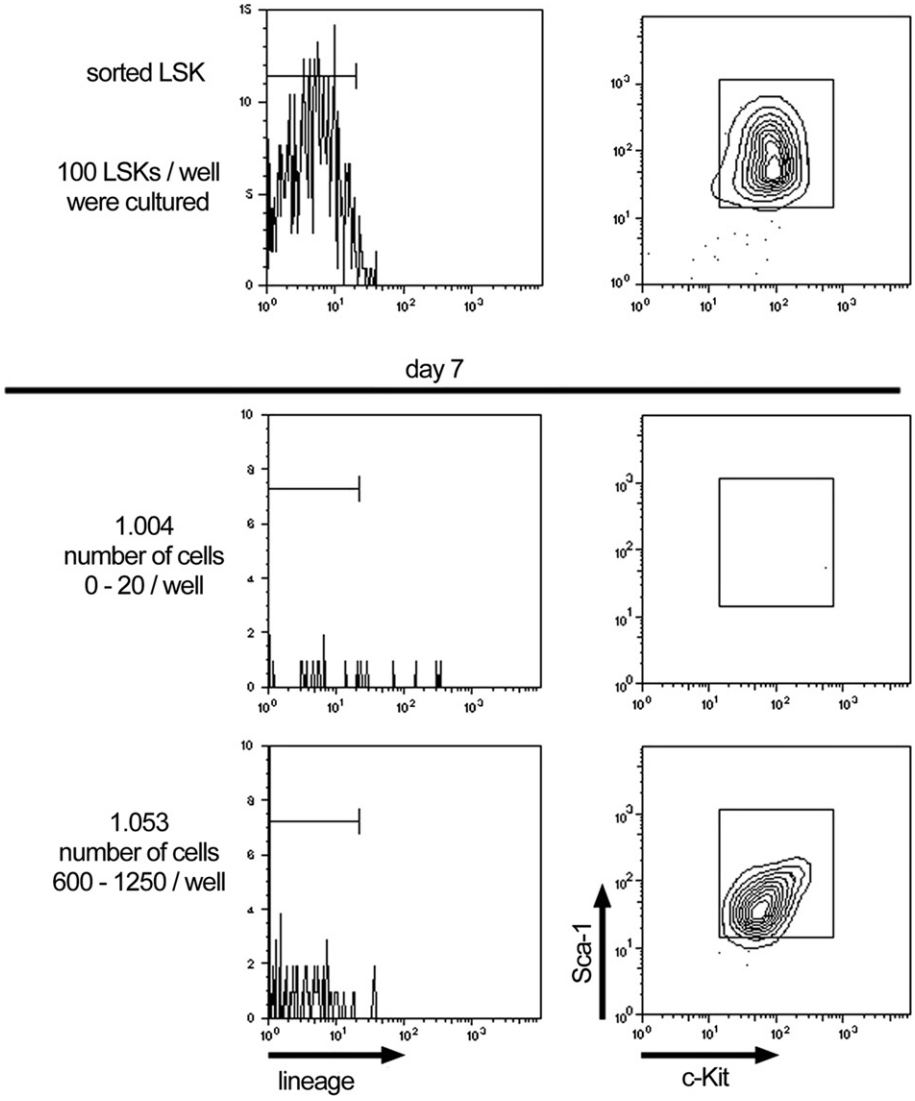

Fig. 3. Characterization of expanded cells. Culturing of the purified LSK. LSK cells were sorted from fresh bone marrow cells, and those having a purity of at least 95% were used for further experimentation. The flow cytometric profile after 7 days of culture is shown. In the lowest-density medium (specific gravity 1.004), no cells remained (upper panel), whereas in the high-density medium (specific gravity 1.053), the number of LSK cells was amplified approximately 10-fold (lower panel).

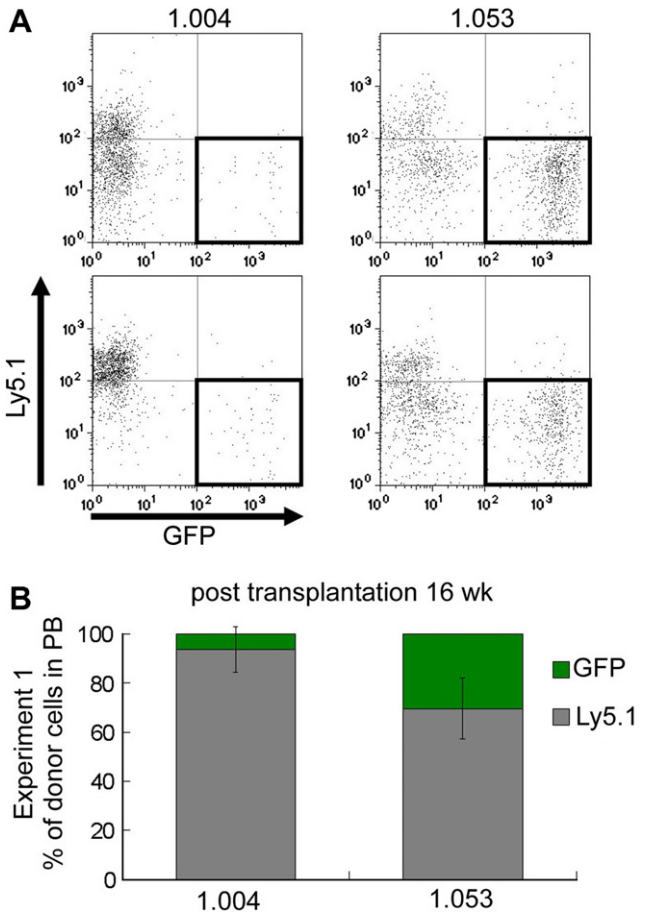

Fig. 4. Bone marrow transplantation. (A) A total of 2×10^5 marrow cells from the GFP transgenic mice and the same number of competitor (Ly5.1) cells were transplanted into irradiated (9 Gy) Ly5.2 mice. Sixteen weeks after transplantation, peripheral blood samples were obtained from the recipient mice. The enclosed part in the heavy line shows the donor cells. The marrow cells cultured in the high-density medium (specific gravity 1.053) were retained. (B) In experiment 1, the average donor cell population was increased significantly in this medium. These experiments were performed in triplicate; combined results are summarized in Table 1.

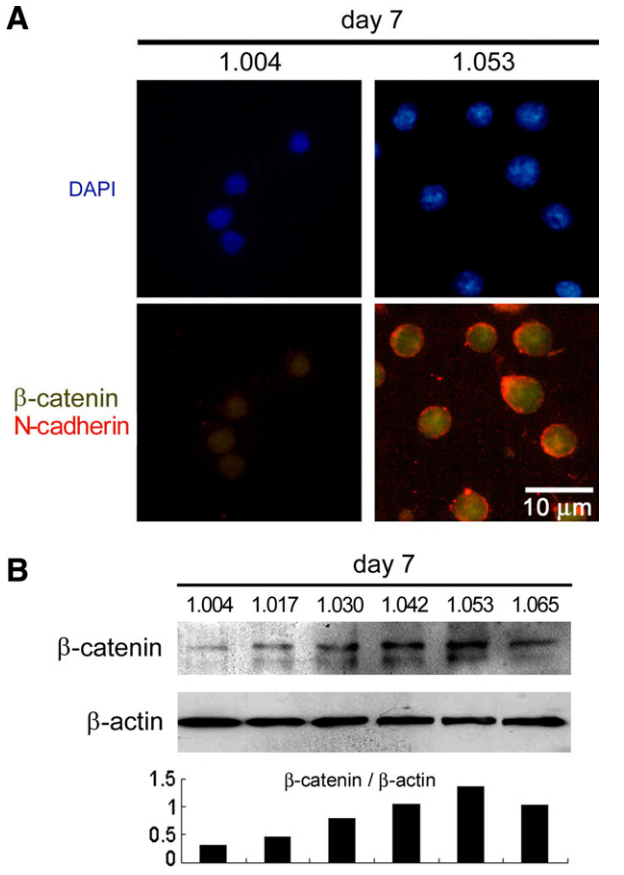

Fig. 5. β-Catenin protein assay. (A) Immunostaining of β-catenin and N-cadherin. After culturing for seven days in the high-density medium (specific gravity 1.053), the expression of N-cadherin (red) on the cell surface and the intranuclear β-catenin (green) was enhanced. (B) Protein assay with Western blotting. The accumulation of β-catenin was the most obvious in this medium.

medium (Fig. 5A). Similar results were obtained for the total cell population on the seventh day, as assessed by Western blotting (Fig. 5B). Recently, extracellular signals such as Notch, Wnt, and Hedghog have been implicated in the maintenance of HSCs [7–13]. The Wnt and Notch signaling pathways integrate to regulate HSCs [14], and β-catenin is downstream of Wnt in HSCs. The accumulation of β-catenin therefore implicated Wnt signaling in the mechanism underlying HSC maintenance.

3.4. Long-term culture in the high-density medium

We next examined whether HSCs were amplified to a greater extent by exchanging the culture medium every 7 days. On day

Table 1
The outcomes of transplantation experiments

Experiment	Specific gravity	# of transplanted donor cells	5 weeks	16 weeks	
			% Donor cells in recipients' PB	% Donor cells in recipients' PB	# of positive mice/total # of mice
1	1.004	2×10^5	23.18 ± 16.69	6.33 ± 9.25	2/6
	1.053	2×10^5	59.60 ± 12.80	30.39 ± 12.43	6/6
2	1.004	2×10^5	Not done	0.57 ± 1.11	0/6
	1.053	2×10^5	Not done	19.72 ± 29.37	6/6
3	1.004	2×10^5	12.11 ± 16.18	1.03 ± 2.28	1/6
	1.053	2×10^5	31.50 ± 27.18	11.03 ± 14.42	5/6

Blood cells of the recipients were analyzed at 5 and 16 weeks after transplantation. The number of mice reconstituted with GFP tg cells (positive mice) is the numerator; the number of mice transplanted is the denominator. The positive mice mean percent chimerism was >1.0%.

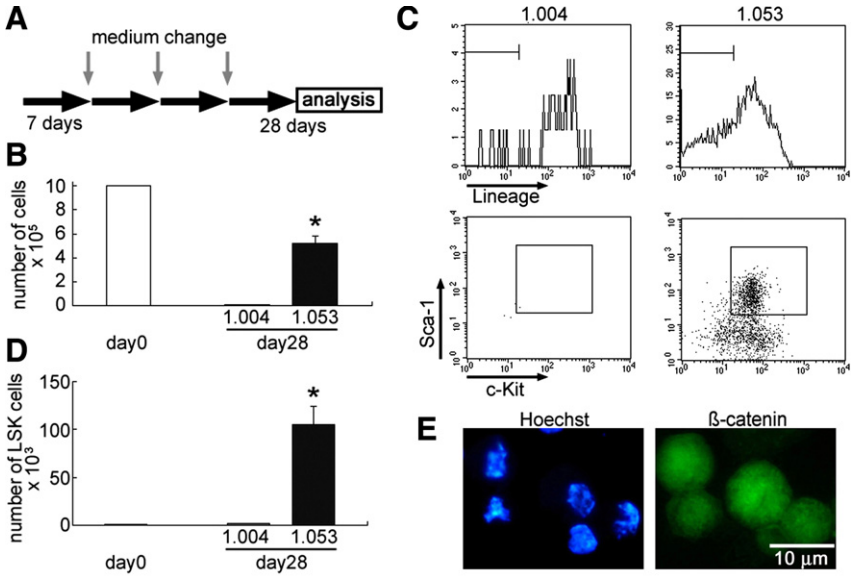

Fig. 6. Culturing by exchanging the medium. (A) Schedule for media exchange. (B) In the high-density medium (specific gravity 1.053), there was only a minimal decrease in the number of cells compared to the initial cell number (1×10^6 cells). (C) Flow cytometric profiles after 28 days of culture. (**D**) The number of LSK cells was amplified by approximately 100-fold compared with fresh marrow. (E) Obvious accumulation of β-catenin with this medium. No staining was observed in the medium with a specific gravity of 1.004, due to the paucity of cells.

28, the high-density medium contained a similar number of cells as in the initial counts, while the number of LSK cells was amplified approximately 100 times (Fig. 6). However, when these cells were transplanted into irradiated mice they no longer had the reconstitution ability of HSCs (data not shown).

4. Discussion

We used a newly developed high-specific-gravity media for the culturing of bone marrow cells from mice. The number of HSCs was amplified in this high-density medium (specific gravity of 1.053). The specific gravity of human blood ranges from 1.050 to 1.060, so the specific gravity of our culture medium was within this range. The cells did not precipitate in this high-density media, and maintenance of HSCs was increased due to accumulation of β-catenin. Phosphorylation of β-catenin contributes to malignant progression and metastasis in cancer cells [15]. Cells adhere to each other via transmembrane cadherin proteins on the cell surface. The cadherin complexes with β-catenin, and via this interaction is bound to the actin cytoskeleton. When β-catenin is phosphorylated by EGF or Src signaling, cancer cell-adhesions are attenuated. However, when phosphorylation of β-catenin is inhibited, cell adhesion is maintained [16]. Thus, when cultured cells do not precipitate, β-catenin is not sequestered by the adhesion complex at the cell surface and on phosphorylation, it translocates into the nucleus. Constitutive β-catenin activation has been linked to loss of HSC hematopoietic differentiation [17], and under conditions where cells are floating, β-catenin could accumulate. In our study, cells cultured for 28 days did not retain the reconstitution ability of HSCs. We propose that this was due to continuous β-catenin activation. On the other hand, mast cells highly express the stem cell antigen Sca-1 and cKit [18]. Marrow cells cultured in a 1.053 g/mL culture system may differentiate into mast cells. Such cells should be referred to as *phenotypical* HSCs, given that they did not retain reconstitution ability in mice after 28 days in culture.

The niche hypothesis, referring to the environment in which HSCs exist, was proposed in 1978 [1]. Subsequently, HSCs were cultured with stromal cells [2]. The niche hypothesis is again topical, and recent reports have implicated osteoblasts as part of the niche [19,20]. It is assumed that cell–cell adhesion is necessary for HSC self-renewal *in vivo*. Although a small quantity of osteoblasts might be included within the marrow cells we used, our results showed an increase in HSC maintenance in an environment with hardly any stromal cells. The cells did not precipitate in media of high specific gravity, and a small area of cell adhesion could be assumed in this situation. There has been no previous research in this field, and furthermore, this study proposes a new technique for maintaining HSC populations for use in regenerative medicine and developmental biology. We anticipate this research contributing significantly to the development of hematology and to the clinical applications of stem cell transplantation.

Acknowledgements: We thank Professor Yoshihiro Takihara for his excellent technical assistance in bone marrow transplantation. The authors have no competing financial interests. GFP tg mice were a kind gift from Dr. M. Okabe (Research Institute for Microbial Diseases, Osaka University).

References

[1] Schofield, R. (1978) The relationship between the spleen colony-forming cell and the haematopoietic stem cell. A hypothesis. Blood Cells 4, 7–25.
[2] Dexter, T.M., Allen, T.D. and Lajtha, L.G. (1977) Conditions controlling the proliferation of haemopoietic stem cells *in vitro*. J. Cell. Physiol. 91, 335–344.
[3] Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T. and Nishimune, Y. (1997) 'Green mice' as a source of ubiquitous green cells. FEBS Lett. 407, 313–319.

[4] Ikuta, K. and Weissman, I.L. (1992) Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. Proc. Natl. Acad. Sci. USA 89, 1502–1506.
[5] Li, C.L. and Johnson, G.R. (1995) Murine hematopoietic stem and progenitor cells: I. Enrichment and biologic characterization. Blood 85, 1472–1479.
[6] Li, C.L., Wu, L., Antica, M., Shortman, K. and Johnson, G.R. (1995) Purified murine long-term *in vivo* hematopoietic repopulating cells are not prothymocytes. Exp. Hematol. 23, 21–25.
[7] Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R. and Weissman, I.L. (2003) A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 423, 409–414.
[8] Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R. and Nusse, R. (2003) Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature 423, 448–452.
[9] Maeno, M., Mead, P.E., Kelley, C., Xu, R.H., Kung, H.F., Suzuki, A., Ueno, N. and Zon, L.I. (1996) The role of BMP-4 and GATA-2 in the induction and differentiation of hematopoietic mesoderm in *Xenopus laevis*. Blood 88, 1965–1972.
[10] Davidson, A.J. and Zon, L.I. (2000) Turning mesoderm into blood: the formation of hematopoietic stem cells during embryogenesis. Curr. Top. Dev. Biol. 50, 45–60.
[11] Bhardwaj, G., Murdoch, B., Wu, D., Baker, D.P., Williams, K.P., Chadwick, K., Ling, L.E., Karanu, F.N. and Bhatia, M. (2001) Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. Nat. Immunol. 2, 172–180.
[12] Zon, L.I. (2001) Self-renewal versus differentiation, a job for the mighty morphogens. Nat. Immunol. 2, 142–143.
[13] Stier, S., Cheng, T., Dombkowski, D., Carlesso, N. and Scadden, D.T. (2002) Notch1 activation increases hematopoietic stem cell self-renewal *in vivo* and favors lymphoid over myeloid lineage outcome. Blood 99, 2369–2378.
[14] Duncan, A.W., Rattis, F.M., DiMascio, L.N., Congdon, K.L., Pazianos, G., Zhao, C., Yoon, K., Cook, J.M., Willert, K., Gaiano, N., Reya, T., Duncan, A.W., et al. (2005) Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat. Immunol. 3, 314–322.
[15] Müller, T., Choidas, A., Reichmann, E. and Ullrich, A. (1999) Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. J. Biol. Chem. 274, 10173–10183.
[16] Kitada, T., Miyoshi, E., Noda, K., Higashiyama, S., Ihara, H., Matsuura, N., Hayashi, N., Kawata, S., Matsuzawa, Y. and Taniguchi, N. (2001) The addition of bisecting *N*-acetylglucosamine residues to E-cadherin down-regulates the tyrosine phosphorylation of beta-catenin. J. Biol. Chem. 276, 475–480.
[17] Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M.M., Birchmeier, W., Tenen, D.G. and Leutz, A. (2006) Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. Nat. Immunol. 10, 1037–1047.
[18] Drew, E., Merkens, H., Chelliah, S., Doyonnas, R. and McNagny, K.M. (2002) CD34 is a specific marker of mature murine mast cells. Exp. Hematol. 30, 1211–1218.
[19] Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., Harris, S., Wiedemann, L.M., Mishina, Y. and Li, L. (2003) Identification of the haematopoietic stem cell niche and control of the niche size. Nature 425, 836–841.
[20] Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., Milner, L.A., Kronenberg, H.M. and Scadden, D.T. (2003) Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425, 841–846.